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A STUDY OF THE CIRCULATING ERYTHROCYTES
OF RAINBOW TROUT, Salmo gairdneri

BY

[illegible]

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA
April [illegible], 1965

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by

Gertrude Hanke Jacoby

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April 15, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate studies for acceptance, a thesis entitled "A Study of the Circulating Iodocompounds of Rainbow Trout, Salmo gairdnerii" submitted by Gertrude Hanke Jacoby in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Repeated injections of NaI^{125} were used to approach a state of isotopic equilibrium in rainbow trout. Thin layer chromatography of untreated plasma revealed increasing quantities of iodotyrosines, thyroxine and triiodothyronine in the plasma. Chromatographic analysis of the hydrolyzates of four thyroid glands indicated that isotopic equilibrium was achieved rapidly in the thyroid. Organic radio iodine was released slowly to the circulation however.

In the presence of large quantities of stable iodide, penetration of radio iodide into the tissues was increased. Saturation of the iodide-binding protein sites in the plasma under these conditions may be responsible for this phenomenon.

ACKNOWLEDGEMENTS

I wish to express my deepest appreciation to Dr. C. P. Hickman, Jr. for his guidance and encouragement throughout this study. I am also indebted to my fellow graduate students, Mr. Brian R. Hammond, Mr. René R. Roth and Mr. Alfred J. Wiggs for much practical advice.

I wish to thank Miss Yoshiko Hirose who assisted me most competently with the laboratory work during the summer of 1964. The Radioisotope Laboratory of the University of Alberta Hospital kindly allowed me the use of their well scintillation counter and sample changer during the early parts of this study.

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I. INTRODUCTION

The circulating iodocompounds have received a very small share of the research attention given to fish thyroid physiology. Chromatographic studies have been carried out on the plasma of the ammocoete and adult of *Lampetra planeri* (Leloup, 1955), *Periophthalmus koelreuteri* (Leloup, 1956), the lungfish *Protopterus annectens* (Leloup, 1958), *Salmo gairdnerii* (Leloup and Fontaine, 1960), the cyclostome *Eptatretus stoutii* (Tong et al, 1961) and the goldfish *Carassius auratus* (Chavin and Bowman, 1965).

The present work was undertaken in the hope of studying the circulating iodocompounds of *Salmo gairdnerii* in a state of isotopic equilibrium, at which point the distribution of radioactivity is identical with the stable iodine picture. Such a state is approached by continued administration of a constant radio iodine/stable iodine supply. The isotope NaI^{125} is well suited for this purpose in having a half life of 60 days and in emitting low energy gamma rays (Daniel et al, 1962). Two experiments were designed, one in which carrier free NaI^{125} alone was administered periodically by injection, and the other in which the radio iodide was accompanied by a known quantity of stable NaI.

Butanol extraction is generally employed before plasma chromatography. It has been shown that butanol does not extract all of the iodotyrosine and iodide from plasma (Ingbar et al, 1954; Robbins et al, 1961). In order to achieve a more reliable picture of the circulating iodocompounds in rainbow trout, it was therefore decided to submit untreated plasma to thin layer chromatography on silica gel.

Taurog has shown that NaI^{131} in contact with filter paper is subject to chemical change when exposed to air drying (Taurog, 1963 c). The

behavior of NaI^{125} on silica gel, alone and in the presence of iodoamino acid standards was therefore studied preliminary to chromatography of radioactive plasma.

The iodocompounds in thyroid hydrolyzates and the penetration of iodine into the tissues were also studied in trout approaching isotopic equilibrium.

II. MATERIALS AND METHODS

A. LIVING MATERIALS

Rainbow trout, Salmo gairdnerii, were obtained from the Province of Alberta fish hatchery in Calgary, Alberta. The fish were held in running dechlorinated water in 124 liter tanks and fed Clark's "New Age" pellet diet. Fish were not fed on days when samples were taken.

B. ADMINISTRATION OF RADIO IODINE

Two groups of fish were given repeated intraperitoneal injections of NaI^{125}. Group A consisted of 39 trout ranging in weight from 40 to 80 g. The fish were acclimated to a temperature of 14 C and a photoperiod of ten hours. The trout were initially injected with 8 μc of carrier-free NaI^{125} in a volume of .04 ml. The fish were re-injected approximately every three days with .02 ml of the original NaI^{125} solution. The first injection was given in the middle of March, 1964 and the experiment lasted for 56 days.

Group B consisted of 40 trout ranging in weight from 20 to 50 g. These fish were held at 18 C and a ten hour photoperiod. The fish were given an initial injection of 1.3 μc NaI^{125} and .144 mg NaI^{127} in a volume of .04 ml. The fish were re-injected with the same volume of the original solution approximately every three days. The experiment lasted 55 days and was conducted in June and July 1964. The trout in both groups suffered heavy mortality during the last days of the experiment, perhaps due to the frequent handling received, and for this reason the experiments had to be terminated.

All injections were done under anaesthesia, a brief immersion in a dilute solution of tricaine methane sulphonate (MS 222, Sandoz Co., Basle). The dose was injected with a 0.25 tuberculin syringe and 27 gauge needle into the posterior part of the coelom through the ventral musculature. A

duplicate of the initial dose was diluted to 100 ml with distilled water and used as a standard.

C. IN VIVO COUNTING OF FISH

Three fish were selected at random daily and an estimate made of their body I^{125} activity. Anaesthetised fish were placed on a moist plastic platform, mounted above a Nuclear Chicago DS 8 low background, wafer crystal surgical scintillation detector such that the region ventral to the anterior insertion of the dorsal fin was located on top of the probe. The standard was counted to determine variation in counter efficiency and physical decay of the original dose.

D. SAMPLING PROCEDURES

After the first injection, three or four fish were sampled at intervals of approximately ten days. A blood sample was obtained by puncturing the heart or ventral aorta with a 22 gauge needle and drawing the blood into a heparinized syringe. The blood was separated in a microcentrifuge and the plasma pipetted into a clean tube.

The fish was then killed, weighed and measured. The thyroid region, consisting of the basibranchial region of the first three gill arches with much of the gill bars, musculature and gill filaments trimmed off, was dissected out and frozen immediately.

Samples of gill, liver, gut, kidney, epaxial trunk muscle, skin and brain tissue were removed, blotted briefly on filter paper, placed in tared counting tubes and weighed. These tissues were digested overnight with 3 ml 6N NaOH and, together with a plasma sample similarly diluted, counted to 4000 counts in a well scintillation counter. To estimate the penetration of I^{125} into these tissues the following ratio was calculated:

$$\frac{T}{P} = \frac{\text{counts per minute of tissue/gram of tissue}}{\text{counts per minute of plasma/gram of plasma}}$$

E. CHROMATOGRAPHY OF PLASMA AND THYROID HOMOGENATES

Thin layer chromatography was employed to separate the iodocompounds of plasma and of digested thyroid tissue. Glass plates 20 x 20 cm were coated with Silica Gel G (Desaga-Brinkman) to a thickness of 500 μ with an adjustable applicator (Desaga-Brinkman, S11). The plates were air dried for approximately 24 hours and then stored in a metal cabinet with a dessicant. Care was taken to use only evenly coated plates.

Samples were applied as nine 3 μl spots at regular intervals, 1.5 cm from the edge of the plate. Several applications were made with air drying in between until 75-100 μl had been deposited on the plate. Samples were always applied on top of 15 μg MIT (3 iodo-L-tyrosine), 15 μg DIT (3:5 diiodo-L-tyrosine), 30 μg T3 (3:5:3 triiodo-L-thyronine), 30 μg T4 (thyroxine) and 10^{-3}M MMI (methyl-mercapto-imidazole). The iodoamino acids were used for purposes of identification and MMI as a necessary reducing agent.

Glass chromatography tanks, large enough to hold two plates, were lined with filter paper saturated with the chromatography solvent, and solvent was added to the bottom of the tank to a height of approximately 1 cm. The plates, held in a metal holder, were placed in the tanks immediately after sample application. The solvents were prepared fresh before each run. The solvents used were 1N butanol:ethanol:6N ammonium hydroxide = 5:2:1 (BEA) and 75% phenol (certified pure for chromatography by Fisher Scientific Company) with 30 mg NaCN added. The plasma samples were generally run only in BEA whereas thyroid samples were spotted on two plates and run in both systems. Plates were removed when the front had advanced at least 13 cm. BEA chromatography required approximately three hours and chromatography in phenol four to five hours.

The amino acid standards were located with a ninhydrin spray and iodide by the starch method for inorganic iodide after the plates had been

thoroughly air dried.

The silica gel was removed from the plates in 1/2 cm strips by means of an improvised metal scraper guided by a plastic marking template included in the Desaga-Brinkman thin layer chromatography kit. Disposable paper funnels were used to guide each silica gel strip into a glass counting tube (Nuclear Chicago TT2). The tubes were counted in a well scintillation counter to 4000 and occasionally 10000 counts.

Plasma was spotted directly on the plates. Thyroids were cut into small pieces and homogenized in a Servall omni-mixer with 5 ml of .9% NaCl, with MMI added to a 10^{-3}M concentration. The homogenate was transferred to a plastic centrifuge tube, centrifuged at 12000 rpm and the supernatant removed. The precipitate was washed two times with 5 ml of .9% NaCl with added MMI.

The extract and washings were combined and incubated for 24 hours at 38 C with 1.5% (w/v) pancreatin (1 USP) in Tris (Tris hydroxymethyl amino methane) buffer at pH 8.5. The digest was then concentrated in vacuo at 38 C by means of a Buchler Rotary Evapo-mix and spotted for chromatography.

III. RESULTS

A. NaI^{125} CHROMATOGRAPHY

1. Effect of air drying on the chromatography of NaI^{125}

Taurog (1963 c) noted that when samples of NaI^{131} were spotted for chromatography, important transformations began immediately and became increasingly evident as the time between application of the sample to filter paper and chromatography increased. Activity appeared in several extraneous bands, indicating the presence of compounds other than NaI^{131}. The principal extraneous band appeared to be identical to the "U2" contaminant found by Taurog (1961) in solutions of NaI^{131} lacking in sufficient reducing agent. Similar contaminants in commercial NaI^{131} solutions were found by Doctor and Trunnell (1959), DeGroot and Berger (1960) and Ahn and Rosenberg (1961).

To determine whether NaI^{125} in contact with thin layer silica gel underwent changes similar to NaI^{131} on filter paper, 3 μl samples of NaI^{125}, with activities of approximately 3000 cpm, were applied 0, 20, 40 and 60 minutes before chromatography with BEA and phenol. Chromatography in BEA showed (Fig. 1, Table 1) that when the sample was spotted and immediately placed in the chromatography tank, 87.7% of the activity appeared as iodide. After 20 minutes iodide accounted for 45% of the activity while 26.7% was concentrated in an extraneous peak moving ahead of iodide and 26% was spread out along the first half of the chromatogram. An increasing proportion of activity was concentrated in the extraneous peak at 40 and 60 minutes. At 60 minutes only 31.9% of the total activity was found as iodide. Another sample, spotted several days later and subjected to 60 minutes of air drying, showed results similar to the 60 minute sample reported above (Fig. 3).

Figure 1. Effect of air drying on NaI^{125} in contact with silica gel prior to chromatography in BEA (butanol:ethanol:6N $NH_4OH = 5:2:1$).

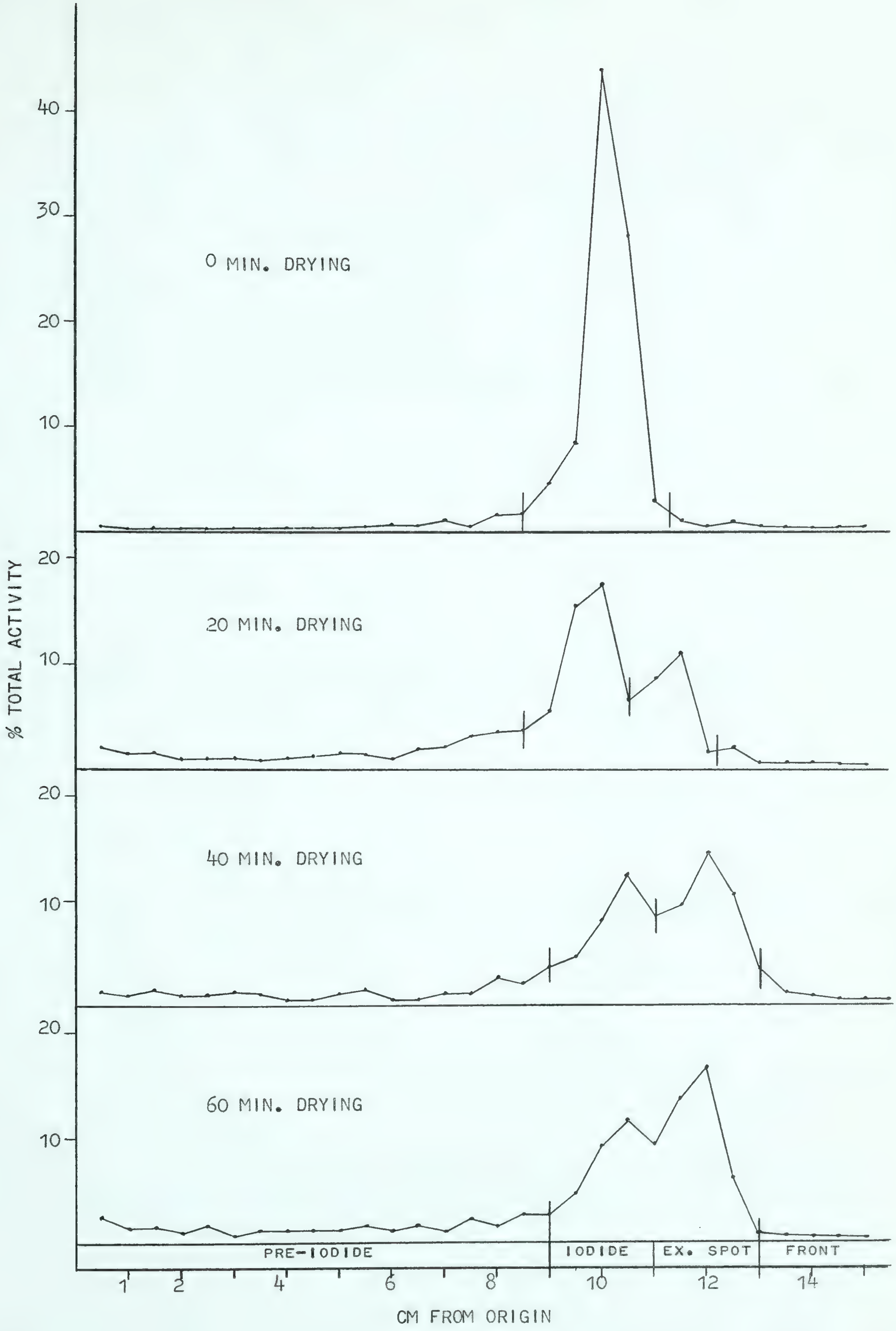

40
30
20
10
0 MIN. DRYING
20
10
20 MIN. DRYING
20
10
40 MIN. DRYING
20
10
60 MIN. DRYING
% TOTAL ACTIVITY
PRE-IODIDE
IODIDE
EX. SPOT
FRONT
1
2
4
6
8
10
12
14
CM FROM ORIGIN

Figure 2. Effect of air drying on NaI^{125} in contact with silica gel prior to chromatography in 75% phenol.

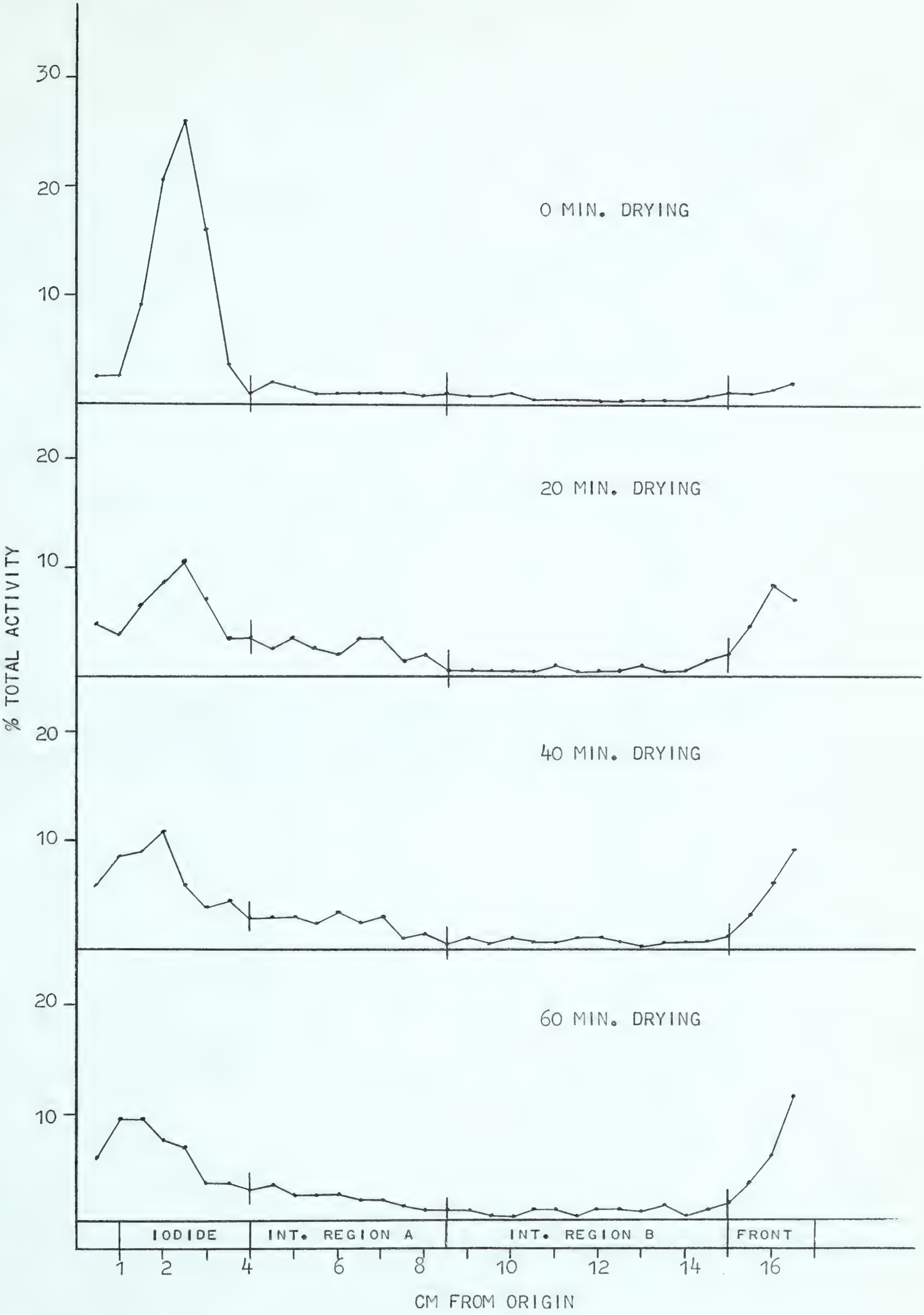
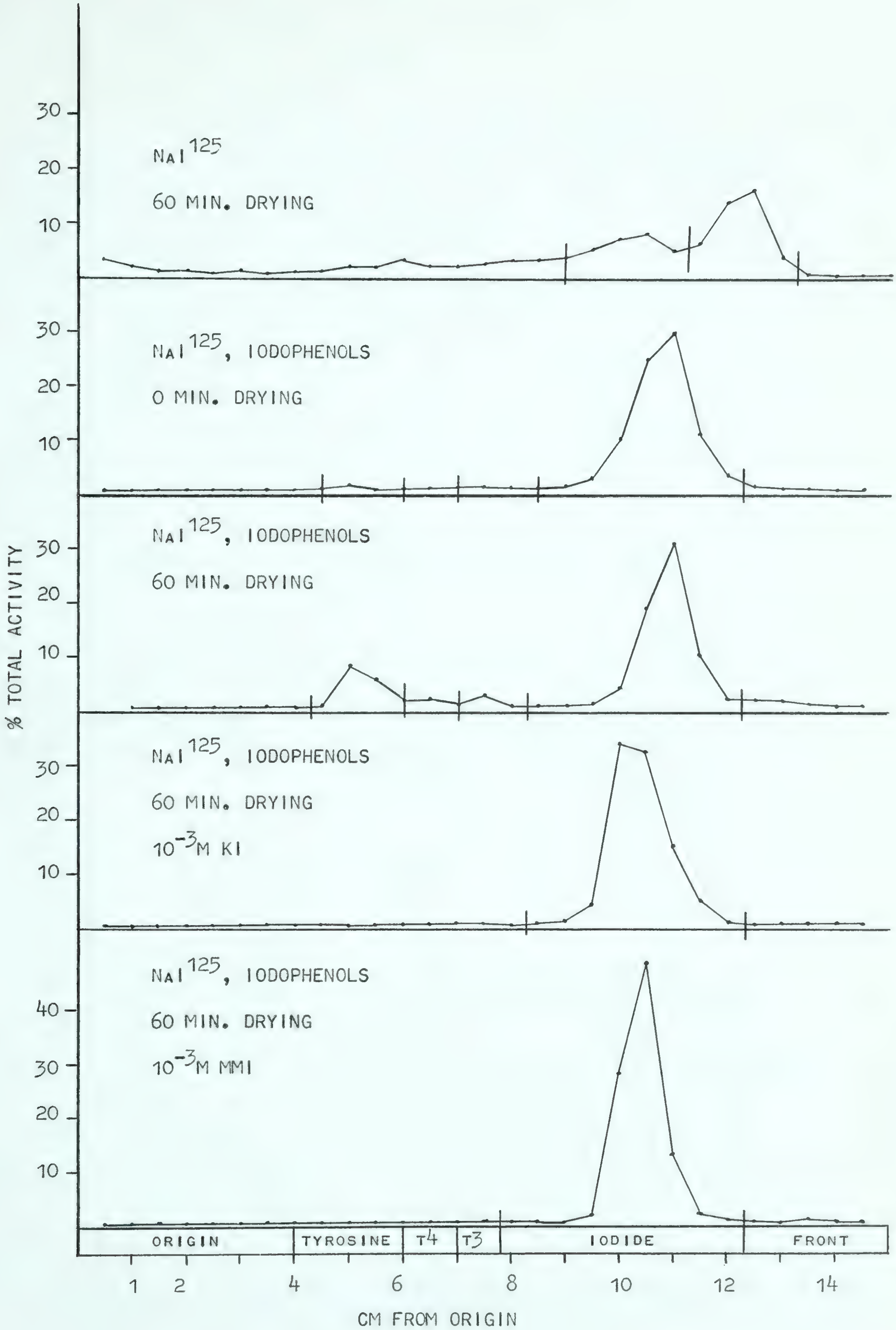

30
20
10
0 MIN. DRYING
20
20 MIN. DRYING
10
% TOTAL ACTIVITY
20
40 MIN. DRYING
10
20
60 MIN. DRYING
10
IODIDE
INT. REGION A
INT. REGION B
FRONT
1
2
4
6
8
10
12
14
16
CM FROM ORIGIN

Figure 3. Labelling of iodoamino acid standards by NaI^{125}. Distribution of activity (% total activity) on silica gel plates spotted with NaI^{125} and iodoamino acid standards. Chromatography in BEA (butanol:ethanol:6N $NH_4OH = 5:2:1$).



NaI^{125}
60 MIN. DRYING
NaI^{125}, IODOPHENOLS
0 MIN. DRYING
NaI^{125}, IODOPHENOLS
60 MIN. DRYING
NaI^{125}, IODOPHENOLS
60 MIN. DRYING
10^{-3}M KI
NaI^{125}, IODOPHENOLS
60 MIN. DRYING
10^{-3}M MMI
% TOTAL ACTIVITY
10
20
30
40
ORIGIN
TYROSINE
T4
T3
IODIDE
FRONT
1
2
4
6
8
10
12
14
CM FROM ORIGIN

Figure 4. Labelling of iodoamino acid standards by NaI^{125}. Distribution of activity (% total activity) on silica gel plates spotted with NaI^{125} and iodoamino acid standards. Chromatography in 75% phenol.

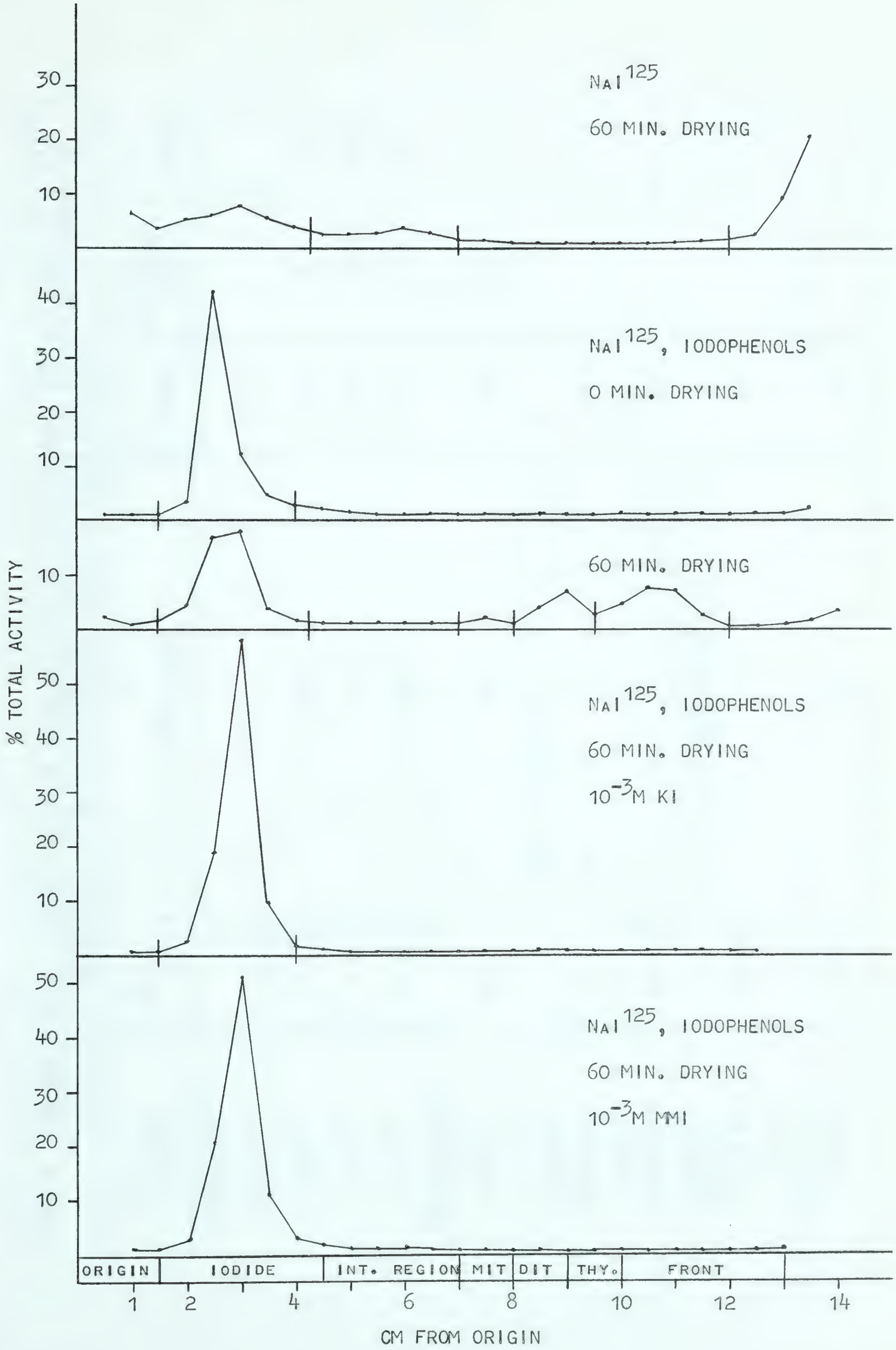

NaI^{125}
60 MIN. DRYING
NaI^{125}, IODOPHENOLS
0 MIN. DRYING
60 MIN. DRYING
NaI^{125}, IODOPHENOLS
60 MIN. DRYING
10^{-3}M KI
NaI^{125}, IODOPHENOLS
60 MIN. DRYING
10^{-3}M MMI
% TOTAL ACTIVITY
10
20
30
40
50
ORIGIN
IODIDE
INT. REGION
MIT
DIT
THY.
FRONT
1
2
4
6
8
10
12
14
CM FROM ORIGIN

Table 1. Distribution of radioactivity (% total activity) on silica gel plates spotted with NaI^{125} alone or in combination with other compounds and exposed to air for varying lengths of time before chromatography in BEA (butanol:ethanol:6N NH$_4$OH = 5:2:1).

<table>
<tr><th>Compounds spotted</th><th>Drying time</th><th colspan="4">Pre iodide</th><th>iodide</th><th>extraneous spot</th><th>front</th></tr>
<tr><td>EXPERIMENT I</td><td></td><td colspan="4"></td><td></td><td></td><td></td></tr>
<tr><td>NaI^{125}</td><td>0 min</td><td colspan="4">7.3</td><td>87.7</td><td colspan="2">5.0</td></tr>
<tr><td>NaI^{125}</td><td>20 min</td><td colspan="4">26.0</td><td>45.0</td><td>26.7</td><td>2.2</td></tr>
<tr><td>NaI^{125}</td><td>40 min</td><td colspan="4">20.9</td><td>37.7</td><td>37.4</td><td>4.0</td></tr>
<tr><td>NaI^{125}</td><td>60 min</td><td colspan="4">24.9</td><td>31.9</td><td>41.8</td><td>1.5</td></tr>
<tr><td>EXPERIMENT II</td><td></td><td colspan="4"></td><td></td><td></td><td></td></tr>
<tr><td>NaI^{125}</td><td>60 min</td><td colspan="4">33.0</td><td>25.8</td><td>39.8</td><td>1.4</td></tr>
<tr><td></td><td></td><td>origin</td><td>tyrosine</td><td>T4</td><td>T3</td><td></td><td></td><td></td></tr>
<tr><td>NaI^{125}
iodophenols</td><td>0 min</td><td>2.4</td><td>2.0</td><td>1.4</td><td>1.7</td><td>88.3</td><td colspan="2">4.1</td></tr>
<tr><td>NaI^{125}
iodophenols</td><td>60 min</td><td>1.8</td><td>16.5</td><td>3.6</td><td>4.2</td><td>68.4</td><td colspan="2">5.6</td></tr>
<tr><td></td><td></td><td colspan="4">Pre iodide</td><td></td><td></td><td></td></tr>
<tr><td>NaI^{125}
iodophenols
10^{-3}M KI</td><td>60 min</td><td colspan="4">3.6</td><td>94.6</td><td colspan="2">1.7</td></tr>
<tr><td>NaI^{125}
iodophenols
10^{-3}M MMI</td><td>60 min</td><td colspan="4">3.1</td><td>95.4</td><td colspan="2">1.5</td></tr>
</table>

Table 2. Distribution of radioactivity on silica gel plates spotted with NaI^{125} alone or in combination with other compounds and exposed to air for varying lengths of time before chromatography in 75% phenol. Distribution expressed as % total activity.

Compounds spotted	Drying time	origin	iodide	int. reg. a	int. region b			front
					MIT	DIT	thyronine	
EXPERIMENT I								
NaI^{125}	0 min	2.7	76.5	8.0		8.9		3.9
NaI^{125}	20 min	4.8	39.8	22.2		11.4		21.7
NaI^{125}	40 min	44.5		26.1		11.1		18.3
NaI^{125}	60 min	43.1		23.5		11.2		22.2
EXPERIMENT II								
NaI^{125}	60 min	8.2	33.3	15.7		9.9		32.9
NaI^{125} iodophenols	0 min	2.6	78.9	8.2	2.3	3.6	2.4	2.0
NaI^{125} iodophenols	60 min	4.5	44.7	5.8	3.1	11.6	23.6	6.7
NaI^{125} iodophenols 10^{-3}M KI	60 min	0.9	89.6	3.9	0.9	1.3	1.7	1.7
NaI^{125} iodophenols 10^{-3}M MMI	60 min	1.5	89.4	3.9	1.1	1.2	0.8	1.9

Phenol chromatography showed (Fig. 2, Table 2) that at 0 minutes, inorganic iodide comprised 76.5% of total activity, a considerably smaller proportion than obtained with BEA. After 20 minutes only 39.8% of the activity remained as iodide, 21.7% was found at the front and 22.2% in a region directly in front of iodide. After 40 and 60 minutes iodide did not move a sufficient distance from the origin to make a clear separation between iodide and origin material, resulting in a high percentage, 43.1%, of total activity composing iodide. In other respects the distribution of activity in the 40 and 60 minute samples closely resemble that of the 20 minute sample. In the second 60 minute sample, run at a different date from those above, iodide and origin material could be easily separated. In this case 33.3% of the activity was iodide with 32.9% of the activity at the front and 15.7% in the region preceeding iodide (Fig 4).

A comparison of BEA and phenol solvents shows that at 0 minutes iodide held 11% less of the total activity after phenol chromatography than after BEA chromatography. The transformation of radio iodide into extraneous compounds is most probably the result of oxidation (Taurog, 1963 c). Phenol is a strong oxidizing agent that seems to accelerate the transformation reactions that occur immediately following sample application. However, the effect of phenol becomes less important with time.

2. NaI^{125} labelling of iodoamino acid carriers

During preliminary experiments it was found that, when radioactive plasma samples were chromatogrammed together with iodophenol standards, very noticeable amounts of tagged iodoamino acids seemed present in the plasma. In the absence of standards however, only small amounts or no iodoamino acids were apparent. The plasma radioactive iodide was apparently tagging the standards and thus giving an erroneous picture.

It seemed worthwhile to determine if this phenomenon was brought about by air drying of a sample in contact with silica gel. Taurog found that labelled iodophenols (Taurog, 1963 a) and thyroid extracts (Taurog, 1963 b) deiodinate when dried on filter paper, glass paper and thin layer silica gel. These supposedly inert substances seem to create an environment susceptible to action of unknown factors in air on radio iodide and iodinated amino acids (Taurog, 1963 a). It seems likely that exchange between radioactive inorganic iodide and organic iodine compounds is a reaction also facilitated by these circumstances. It is known that in this kind of exchange, oxidation of iodide to iodine is a preliminary step in the reaction (Gross, 1954).

To investigate the effect of iodoamino acid carriers on the chromatography of NaI^{125}, NaI^{125} was spotted on top of a mixture of MIT, DIT, T3 and T4 and exposed to air for 0 and 60 minutes prior to chromatography.

Chromatography in BEA showed (Fig. 3, Table 1) that at 0 minutes 88.3% of the activity was iodide. After 60 minutes of drying iodide accounted for 68.4% of the total activity, the tyrosines 16.5%, T4 3.6% and T3 4.2%. NaI^{125} run without carriers after 60 minutes of drying showed only 25.8% activity in the iodide peak with the extraneous compound taking up 39.8% and 33% spread out behind the iodide peak. The extraneous compound was present to only a very small extent (4%) in the sample run with carriers. The carriers thus seem to exert a stabilizing influence on the iodide. Activity in the region behind iodide was considerable in both samples but in the sample containing iodophenols the activity was concentrated in these compounds, especially in the iodotyrosines.

The same general conclusions may be drawn from the sample chromatogrammed in phenol (Fig. 4, Table 2). After 0 minutes 78.9% of the activity was iodide. After 60 minutes the sample with carriers had an iodide peak

comprising 44.7% of the total activity with much activity concentrated in the iodophenols. In the sample without carriers 33.3% of the activity was iodide and 32.9% extraneous compound located at the front. The stabilizing effect of the standards is again evident.

Taurog found that the addition of KI or MMI to the sample before air drying virtually eliminated the formation of extraneous compounds by NaI^{131} (Taurog, 1963 c) and the deiodination of labelled iodophenols (Taurog, 1963 a) and thyroid extracts (Taurog, 1963 b). To study the effect of these compounds on the labelling of iodophenols noted above, 10^{-3}M MMI or 10^{-3}M KI were included in samples of NaI^{125} with iodophenol carriers and the plates were air dried for 60 minutes. Both compounds greatly reduced any labelling of the carriers, appearance of distinct extraneous substances or smearing of activity along the chromatogram. After BEA chromatography (Fig. 3, Table 1) 94.6% and 95.4% of the activity was iodide when 10^{-3}M KI and 10^{-3}M MMI were included respectively. After phenol chromatography (Fig. 4, Table 2) 89.6% and 89.4% of the activity was iodide with KI and MMI respectively.

It may be concluded that in the presence of iodophenol standards radioactive iodide will label these standards if left in contact with silica gel for any length of time. Even when the sample is applied and the plate is placed in the chromatography tank immediately, some radioactivity appears in these compounds. Inclusion of 10^{-3}M KI or 10^{-3}M MMI in the sample will effectively stabilize the NaI^{125} and prevent this exchange labelling.

MMI is a strong reducing agent. It is known that the oxidation of iodide to iodine is a necessary prerequisite to exchange labelling of iodophenols by radioactive iodide (Gross, 1954). This oxidation apparently occurs rapidly when NaI^{125} is applied to silica gel. The effectiveness of MMI in the prevention of exchange labelling may lie in the blocking of this step.

B. CHROMATOGRAPHIC SEPARATION OF THE IODOCOMPOUNDS OF PLASMA

After an initial application of reference standards and MMI, plasma samples were spotted directly on silica gel plates and chromatogrammed in BEA. Distribution of radioactivity along the chromatogram was calculated as percent of total activity. The results are shown in Table 3 for fish of Group A and Table 4 for fish of group B. The mean percents of activity found in the iodotyrosine, T4, T3 and iodide sections of the chromatograms as well as the mean total organic iodine and hormonal iodine were calculated for each sampling period (Table 5, Figure 5).

1. Experiment A

The mean percent of total radioactivity found as organic iodine was less than one half percent after ten days of injection and increased gradually to about four and one-half percent at fifty days (Fig. 5, Table 5). There is considerable variability among individual fish within each sampling period, mainly caused by the range in values in the T3 fraction. The amount of radioactive T3 is in all probability falsely high for some of the plasma samples (Table 3: # 6, 12, 13, 15, 16) due to the inadequate separation of T3 and iodide in these chromatograms and consequent inclusion of iodide in T3 activity. These chromatograms are excluded in the corrected curves for T3, hormonal iodine and total organic iodine in Figure 5. Using the corrected curve for T3, T4 and T3 are found in about equal proportion in the plasma, the mean value of each reaching a maximum of about 1.75% after 50 days of injections.

Radioactive MIT and DIT possess less than one half percent of the total activity until the 40 day sampling period and then account for roughly one to one and one-half percent of the activity. Although this is a small proportion of the total radioactivity, it represents about one third of the

Table 3. Distribution of radioactivity (% total activity) along silica gel plates spotted with 50-100 µl plasma and chromatogrammed in BEA (butanol:ethanol:6N NH_4OH=5:2:1). Group A.

Fish no.	Day	origin	int. reg.	iodo-tyrosine	int. reg.	T4	int. reg.	T3	int. reg.	iodide	front	total %
1	10	.3	.3	.1		.1		.1		98.6	.4	99.9
2	10	.05	.4	.05		.2		.2	.2	98.6	.3	100.0
3	10	.1		.2		.1		.1	.2	99.4	.1	100.2
5	20	2.1	.1	.1		.3		.1	.3	96.6	.3	99.9
6	20	2.9	.6	.6		.8		2.2 *		92.6	.3	100.0
7	20	.7	.1	.3		.3		.3	.3	97.7	.2	99.9
8	20	4.5	.4	.5		.3		.2	.6	92.4	1.1	100.0
11	30	.3	.3	.2		.6	.2	.9	.3	95.9	1.4	100.1
12	30	1.2	.2	.4		.7	.5	1.9 *		94.4	.7	100.0
13	30	.2	.3	.4	.6	.6		2.3 *		94.6	1.1	100.0
14	40	1.2	1.9	1.8		.7		.5	1.3	88.8	3.7	99.9
15	40	.1	.3	.5		.7	.2	3.2 *		94.2	.8	100.0
16	40	.8	.3	1.3	.9	1.1	.3	4.4 *	2.1	86.8	2.0	100.0
17	40	.6	.1	1.1	.2	.7		.8	.6	93.5	2.2	99.8
18	50	.1	1.1	.6		1.1		1.2		92.6	3.3	100.0
19	50	1.4	1.0	1.0		2.9		2.6	3.4	85.3	2.2	99.8
20	50	.5	1.0	1.0		1.2	.3	1.6		92.5	1.9	100.0
21	56	1.3	1.1	.2		.2		.2	.1	96.9	.1	100.1
22	56	1.2	.8	.5		.6	.1	1.3	1.0	92.2	2.2	99.9
23	56	2.4	4.2	3.1		.9		1.2		86.5	1.7	100.0
24	56	2.0	1.8	2.4	.7	1.4		2.2		87.9	1.7	100.1

* Unreliable values due to inadequate separation of T3 and iodide.

Table 4. Distribution of radioactivity (% total activity) along silica gel plates spotted with 50-100 µl plasma and chromatogrammed in BEA (butanol:ethanol:6N NH_4OH 5:2:1). Group B.

Fish no.	Day	origin	int. reg.	iodo-tyrosine	int. reg.	T4	int. reg.	T3	int. reg.	iodide	front	total %
31	10		1.4	1.4		1.8	.7	2.2		89.2	3.4	100.1
32	10		.2	1.6		.2		.4		97.3	.2	99.9
33	10		7.6	.4		1.2		.8	.2	87.3	2.5	100.0
34	19		2.4	1.0		1.0	.5	1.5		91.4	2.2	100.0
42	19		2.2	.9		---		1.1	.7	89.9	5.2	100.0
46	28		3.9	2.7		1.0		.9	1.5	87.4	2.5	99.9
47	38		3.0	.9		1.5		1.8	2.4	87.7	2.7	100.0

Figure 5. Mean values (% total activity) ± S.E. of the circulating iodo-compounds separated by thin layer chromatography with BEA (butan ethanol:6N $NH_4OH = 5:2:1$) as solvent. The range is marked for 56 days. Unreliable T3 values are omitted in the corrected curves for T3, hormonal iodine and total organic iodine. Exp. A

- - - - - corrected curves.

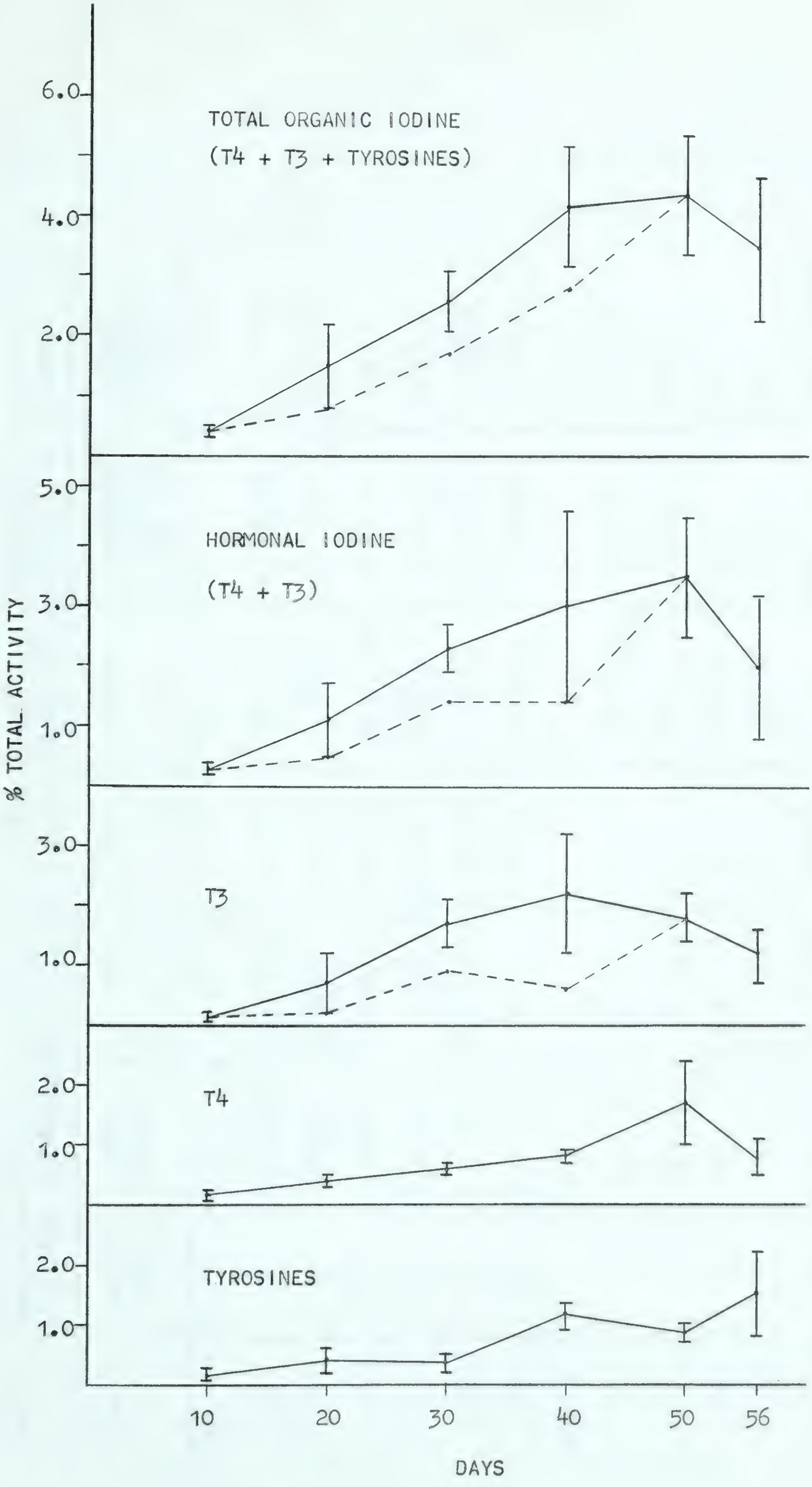

6.0
4.0
2.0
TOTAL ORGANIC IODINE
(T4 + T3 + TYROSINES)
5.0
3.0
1.0
HORMONAL IODINE
(T4 + T3)
% TOTAL ACTIVITY
3.0
1.0
T3
2.0
1.0
T4
2.0
1.0
TYROSINES
10
20
30
40
50
56
DAYS

Table 5. Mean values (% total activity) ± S.E. of the circulating iodocompounds separated by thin layer chromatography with BEA (butanol:ethanol:6N NH_4OH=5:2:1) as solvent.

Day	Sample size	iodo-tyrosine	T4	T3	iodide	hormonal iodine (T4 T3)	total organic iodine
EXP. A							
10	3	.12±.04	.13±.03	.13±.03	98.7 ± .3	.3 ± .07	.4 ±.05
20	4	.38±.21	.42±.12	.7 ± .5	94.8±1.4	1.1 ± .6	1.5 ± .7
30	3	.33±.07	.63±.04	1.7 ± .4	95.0 ± .5	2.3 ± .4	2.6 ± .5
40	4	1.2 ± .2	.8 ± .1	2.2±1.0	90.8±1.8	3.0±1.6	4.2±1.0
50	3	.9 ± .1	1.7 ± .7	1.8 ± .4	90.1±1.8	3.5±1.0	4.4±1.0
56	4	1.5 ± .7	.8 ± .3	1.2 ± .4	90.9±2.5	2.0±1.2	3.5±1.2
EXP. B							
10	3	1.1 ± .4	.7 ± .5	1.1 ± .5	91.3±3.1	1.8±1.0	2.9±1.0
19	2	.9	.5	1.3	90.6	1.8	2.7
28	1	2.7	1.0	.9	87.4	1.9	4.6
38	1	.9	1.5	1.8	87.7	3.3	4.2

total organic iodine. Its abundance in the plasma varies considerably from fish to fish and no definite relationship between it and the hormonal iodine component is evident.

2. Experiment B

The plasma of Group B was considerably less radioactive than the plasma of Group A and in some cases the total activity of a chromatogram was judged too low to give a reliable picture of the I^{125} distribution. The chromatograms with a total activity of at least 2000 counts per minute are analyzed in Table 4 and the mean values of the various iodine fractions are found in Table 5.

At 10 days a mean three percent of the activity was found as organic iodine and approximately two thirds of this was hormonal iodine. The distribution of activity remained essentially the same at 19 days. At 38 days about four percent of the activity was organic iodine, the distribution of activity being similar to that at 40 days in Group A. Organic iodine thus appeared more quickly in the plasma of the Group B trout but the final distribution reached was approximately the same in the two groups.

C. CHROMATOGRAPHIC SEPARATION OF THYROID HYDROLYZATES

Four thyroids from Group A were extracted with .9% NaCl and digested with pancreatin before chromatography in BEA and phenol. One thyroid was divided into three parts and digested for 9, 22 and 70 hours before chromatography and the other three thyroids were digested for 24 hours. BEA and phenol chromatograms for thyroid 2, sampled at 10 days, are shown in Figure 6 and the distribution of activity, expressed as percent of total activity, in the different fractions of the chromatograms is shown in Table 6.

1. Chromatography in BEA and phenol

By chromatography in BEA it was possible to separate the iodotyrosines,

Figure 6. Distribution of activity among the iodoamino acids in thyroid tissue. Fish no. 2, 10 days of NaI^{125} injections, 24 hours proteolytic hydrolysis, Experiment A. Chromatography in BEA (butanol:ethanol:6N NH_4OH 5:2:1) and 75% phenol.

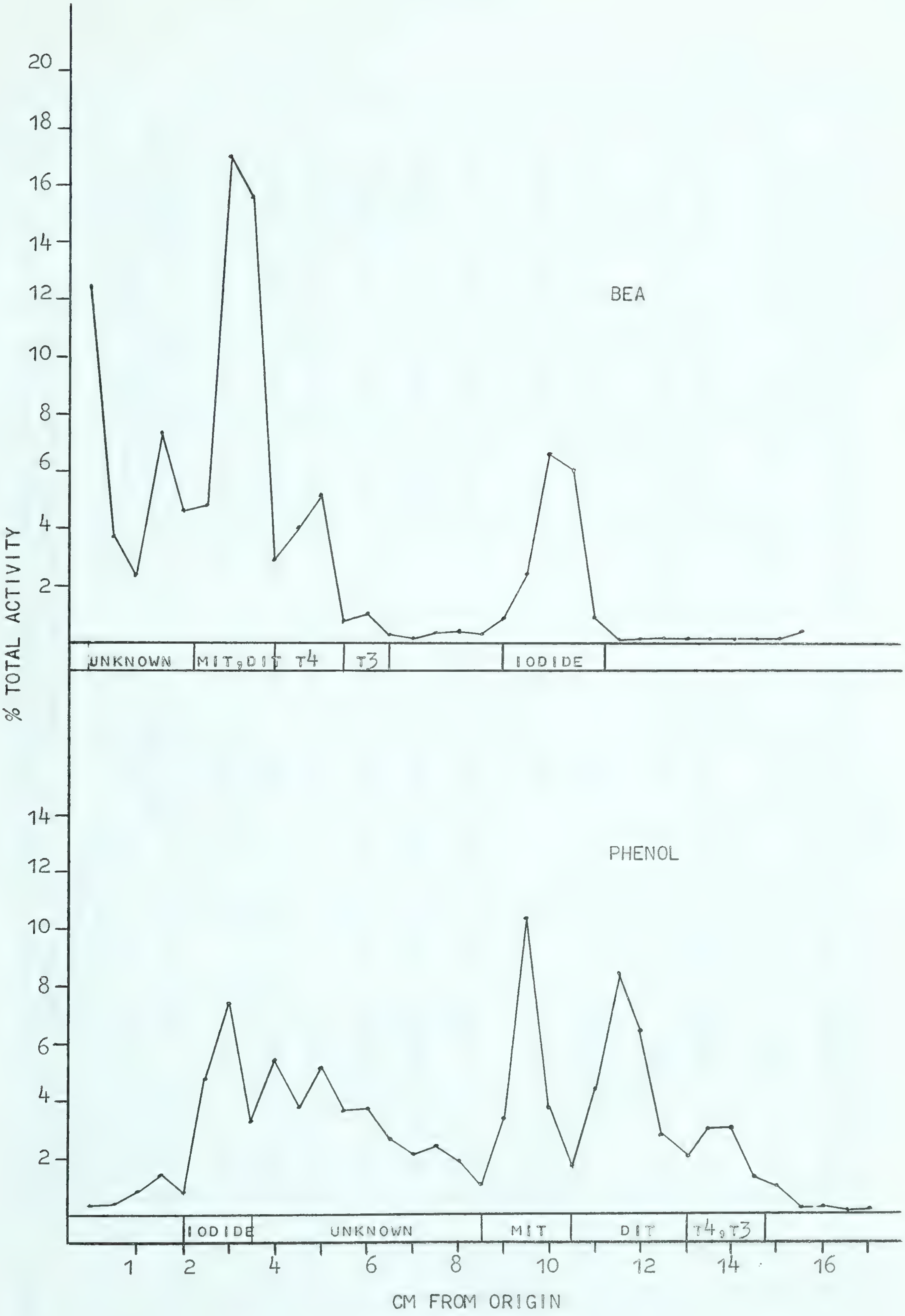

BEA
PHENOL
% TOTAL ACTIVITY
20
18
16
14
12
10
8
6
4
2
UNKNOWN
MIT, DIT
T4
T3
IODIDE
14
12
10
8
6
4
2
IODIDE
UNKNOWN
MIT
DIT
T4, T3
1
2
4
6
8
10
12
14
16
CM FROM ORIGIN

Table 6. Distribution of activity (% total activity) among the different fractions of thyroid hydrolyzates, separated by thin layer chromatography with BEA (butanol:ethanol: 6N NH_4OH=5:2:1) and 75% phenol.

Fish no.	Day	Digt. time	Solvent	MIT	DIT	iodo-tyrosine	T4	T3	iodo-thyron.	org. iodine	iodide	ext. material
1	10	9 h.	BEA			38.8	10.3	1.8	12.1	50.9	17.8	31.3
1	10	22 h.	BEA			41.5	11.8	1.2	13.0	54.5	13.3	32.2
1	10	70 h.	BEA			40.7	9.1	2.0	11.1	51.8	14.4	33.8
1	10	9 h.	phenol	31.5	18.9	50.4			7.8	58.2	11.3	30.4
1	10	22 h.	phenol	17.8	26.9	44.7			14.2	58.9	16.5	24.6
1	10	70 h.	phenol	20.0	23.2	43.2			11.8	55.0	19.6	25.4
2	10	24 h.	BEA			38.7	11.1	1.3	12.4	51.1	17.0	31.9
2	10	24 h.	phenol	19.0	23.3	42.3			7.9	50.2	14.2	35.6
9	20	24 h.	BEA			37.2	9.1	2.2	11.3	48.5	3.8	47.4
9	20	24 h.	phenol	20.7	24.7	45.4			4.6	49.3	50.7	
14	30	24 h.	BEA			34.1	6.3	1.6	7.9	42.0	6.2	51.8
14	30	24 h.	phenol	17.3	24.0	41.3			6.0	47.3	17.6	35.0

T4, T3 and iodide. Phenol chromatography separated MIT and DIT from the iodothyronines and iodide (Fig. 6). Both solvents revealed considerable quantities of material in the hydrolyzates that could not be identified with iodide or any of the iodoamino acid standards chromatogrammed together with the thyroid extract. After BEA chromatography this material separated into two distinct peaks, one at the origin and one immediately following the iodotyrosines. The unknown material preceeded iodide after phenol chromatography and did not always separate completely from the iodide peak. For this reason the iodide values obtained after BEA chromatography are probably more reliable. The total iodotyrosine and iodothyronine values after chromatography in the two solvents were comparable (Table 6).

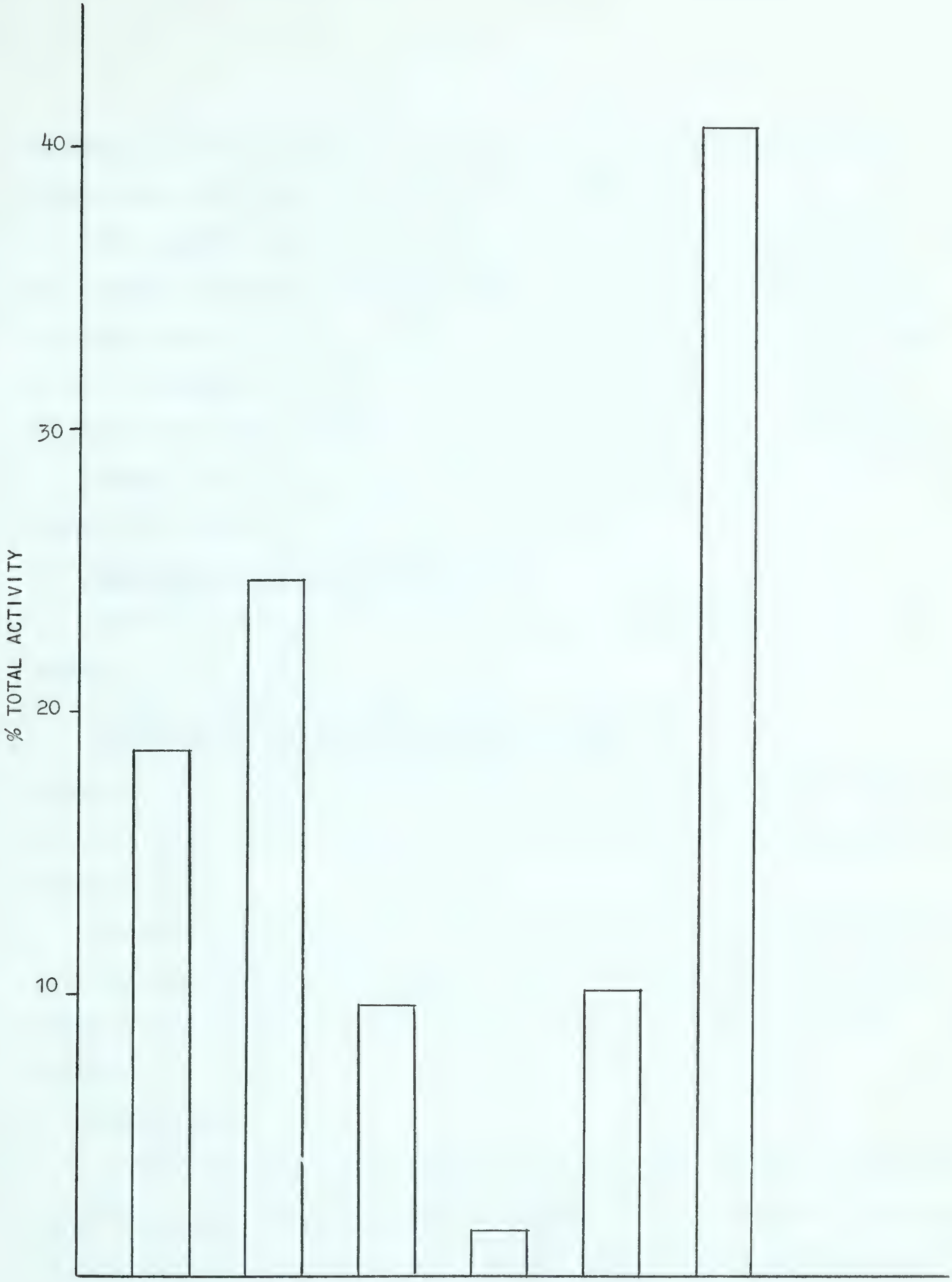
2. Effect of hydrolysis time on distribution of activity

Tong et al (1963) have shown that prolonged digestion of rat thyroids with pancreatin results in the deiodination of T4 and suggest that digests should be tested during the course of digestion to determine at which point maximal T4 yields occur. Aliquots of one thyroid were digested for 9, 22 and 70 hours. The distribution of activity among the different thyroid fractions after chromatography in BEA changed relatively little with increased digestion time (Table 6). The maximum yields of both iodotyrosines and iodothyronines occurred after 22 hours at which time the iodide fraction was also the lowest. Chromatography in phenol also showed that the maximum amounts of organic iodine were recovered after 22 hours but the proportion of MIT to DIT changed considerably with time.

3. Distribution of activity among iodoamino acids in thyroid tissue

The thyroids sampled at 10 days showed similar distributions of activity (Table 6). Approximately 50-55% of the total activity was organic iodine, 15% iodide and 30-35% unknown material. T4 accounted for 11-12% of the

Figure 7. Average distribution of activity (% total activity) among the fractions of four thyroid hydrolyzates. MIT, DIT calculated from phenol chromatograms. T4, T3, iodide and unknown calculated from BEA (butanol:ethanol:6N NH_4OH) chromatograms.


% TOTAL ACTIVITY
40
30
20
10
MIT
DIT
T4
T3
IODIDE
UNKNOWN

activity, T3 less than 2% and MIT and DIT 40-45%, with DIT taking up the larger share after 22-24 hours of pancreatin digestion.

The thyroids sampled at 20 and 30 days showed a larger proportion of the activity incorporated into the unknown material and thus less activity as organic iodine. Iodide accounted for 3.8% at 20 days and 6.2% at 30 days. T4 and T3 accounted for 11% and 7% of the activity at 20 and 30 days respectively while the iodotyrosines accounted for 35-45% of the total activity.

Figure 7 shows the average distribution of activity of MIT, DIT, T4, T3, iodide and unknown in the four thyroids studied.

D. PENETRATION OF IODIDE INTO THE TISSUES

Penetration of radioactive iodine from the plasma into the tissues was measured by the ratio:

$$\frac{\text{counts per minute of tissue / gram of tissue}}{\text{counts per minute of plasma / gram of plasma}} = \frac{T}{P}$$

Ratios for gill, liver, gut, kidney, epaxial trunk muscle, skin and brain of fish of Groups A and B, sampled at approximately ten day intervals, are plotted in Figure 8.

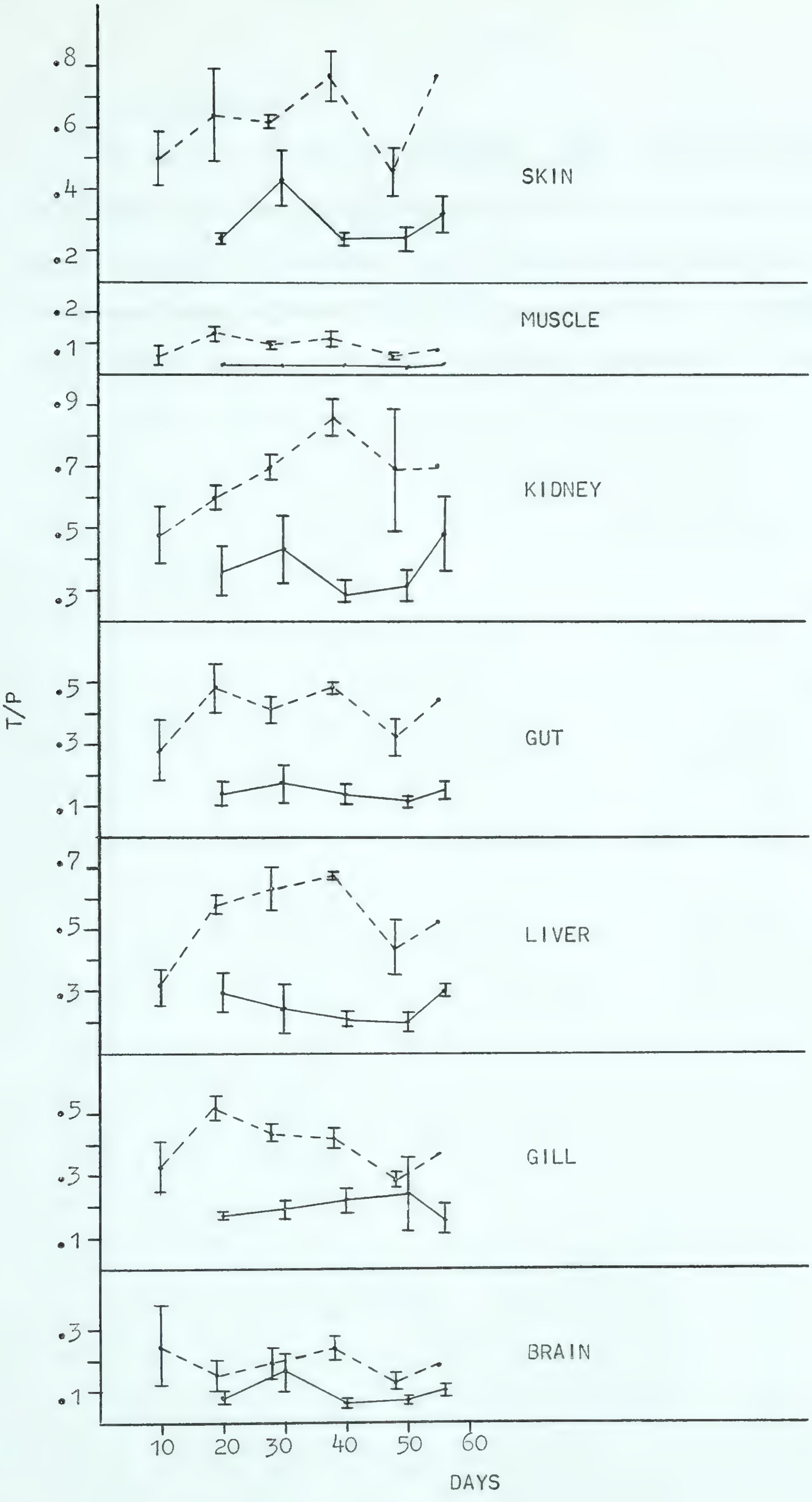
Penetration of radioactive iodine into the tissues of Group B is greater than the penetration into the tissues of Group A. The overall means for each tissue of the two groups are significantly different at the 1% level of probability.

1. Experiment A

The T/P ratios of Group A change little with time. Increased penetration of I^{125} into skin, kidney and brain is evident at 30 days after the beginning of the injection program, followed by a drop back to the original level at 40 and 50 days and another rise at 56 days. T/P ratios for gill, liver, gut and muscle remain relatively constant.

Figure 8. Penetration of I^{125} into the tissues. Mean T/P (tissue radioactivity/plasma radioactivity) plus, minus S.E. The range is marked for day 56 in Experiment A and for day 48 in Experimen

——— Experiment A
– – – – Experiment B


.8
.6
.4
.2
SKIN
.2
.1
MUSCLE
.9
.7
.5
.3
KIDNEY
T/P
.5
.3
.1
GUT
.7
.5
.3
LIVER
.5
.3
.1
GILL
.3
.1
BRAIN
10
20
30
40
50
60
DAYS

2. Experiment B

In addition to being consistently higher, the T/P ratios of Group B vary more with time than do those of Group A. The ratios for brain and muscle change little while those for liver, gut, kidney and skin show increased penetration of I^{125} until a maximum value is reached at 38 days followed by a drop at 48 days and another increase at 55 days.

IV. DISCUSSION

A. THE CIRCULATING IODOCOMPOUNDS

1. Methods based on the protein-bound iodine (PBI) determination

Chemical methods have most frequently been used to determine the quantities of plasma inorganic and hormonal iodine. The plasma protein-bound iodine (PBI) has commonly been considered a measure of the amount of hormonal iodine circulating in the plasma. Plasma total iodine and PBI values of cyclostomes, selachians, teleosts and lungfish are reviewed by Leloup and Fontaine (1960).

T4 is bound to plasma proteins in all vertebrates studied (Pitt-Rivers and Tata, 1959). A thyroxine-binding protein (TBP) has been demonstrated in plaice and brown trout, but the binding has been found to be not complete in the salmon and the carp as evidenced by the slight penetration of T4 into the red blood cells of these species (Leloup and Fontaine, 1960). T3 is bound less firmly to plasma proteins than is T4.

Hickman (1962) showed that in the starry flounder (Platichthys stellatus), serum PBI increased with increasing inorganic iodide concentrations while the butanol-extractable iodine (BEI), which separates protein bound as well as free T4 and T3 from inorganic iodide and the iodotyrosines, remained constant. Binding of iodotyrosines or inorganic iodide to plasma proteins was apparently occurring in this case. Leloup and Fontaine (1960) have shown that in the salmonids iodide is largely bound to plasma proteins. This binding is, according to Leloup and Fontaine (1960), destroyed by the trichloracetic acid precipitation of proteins used in PBI determinations. Binding of iodotyrosines could therefore explain the difference in PBI and BEI values. The possibility of iodotyrosines being bound to plasma proteins and the lack of complete binding of T4 and T3 make the PBI value a less than perfect indicator

of the quantity of circulating thyroid hormone in fish. The BEI determination offers a better alternative (Hickman, 1962).

The conversion ratio method, adapted by Hickman (1961) to analysis of small quantities of plasma, measures the relative distribution of radioactive organic and inorganic iodine in the plasma.

$$\text{C.R.} = \frac{\text{PBI count per minute} \times 100}{\text{PBI count per minute} + \text{iodide count per minute}}$$

$$= \frac{\text{organic radio iodine}}{\text{total plasma radioactivity}}$$

Conversion ratios have been used to study iodine metabolism in Salmo gairdnerii and Platichthys stellatus (Hickman, 1961), juvenile steelhead trout (Eales, 1963 b), juvenile Oncorhynchus (Eales, 1961, 1963 a) and three spine stickleback (Wiggs, 1963). This method, in using the PBI determination, assumes that T4 and T3 are completely bound to plasma protein and that the tyrosines and iodide are not bound and is therefore subject to the criticisms offered above.

2. Chromatographic separation of the iodocompounds of plasma

a. Chromatography of butanol extracts of plasma

Identification of the circulating iodocompounds is possible by means of chromatographic analysis. Bowden et al (1955) have shown that the iodophenols can be determined directly by the Ce-As reaction after chromatography. Most frequently however radio iodine methods are used in conjunction with chromatography.

Extraction of a large volume of plasma, often involving a great number of steps, is usually employed before chromatography. Extraction with butanol is most commonly used. It has been found that only about 50% of MIT, 80% of iodide and no thyroglobulin is extracted with butanol (Ingbar et al, 1954; Robbins et al, 1961). A completely true representation of the circulating

iodocompounds is thus not achieved.

Leloup used butanol extracts of plasma and paper chromatography to identify the circulating iodocompounds of the ammocoete and adult of *Lampetra planeri* (Leloup, 1955), *Periophthalmus koelreuteri* (Leloup, 1956), the lungfish *Protopterus annectens* (Leloup, 1958) and *Salmo gairdnerii* (Leloup and Fontaine, 1960). In all cases T4 was identified, with T3 found in smaller quantities. MIT was identified seven days after injection in *Periophthalmus koelreuteri* with hyperfunctioning thyroids. T4 and T3 accounted for 26-56% of the activity and MIT 1.5% (Leloup, 1956).

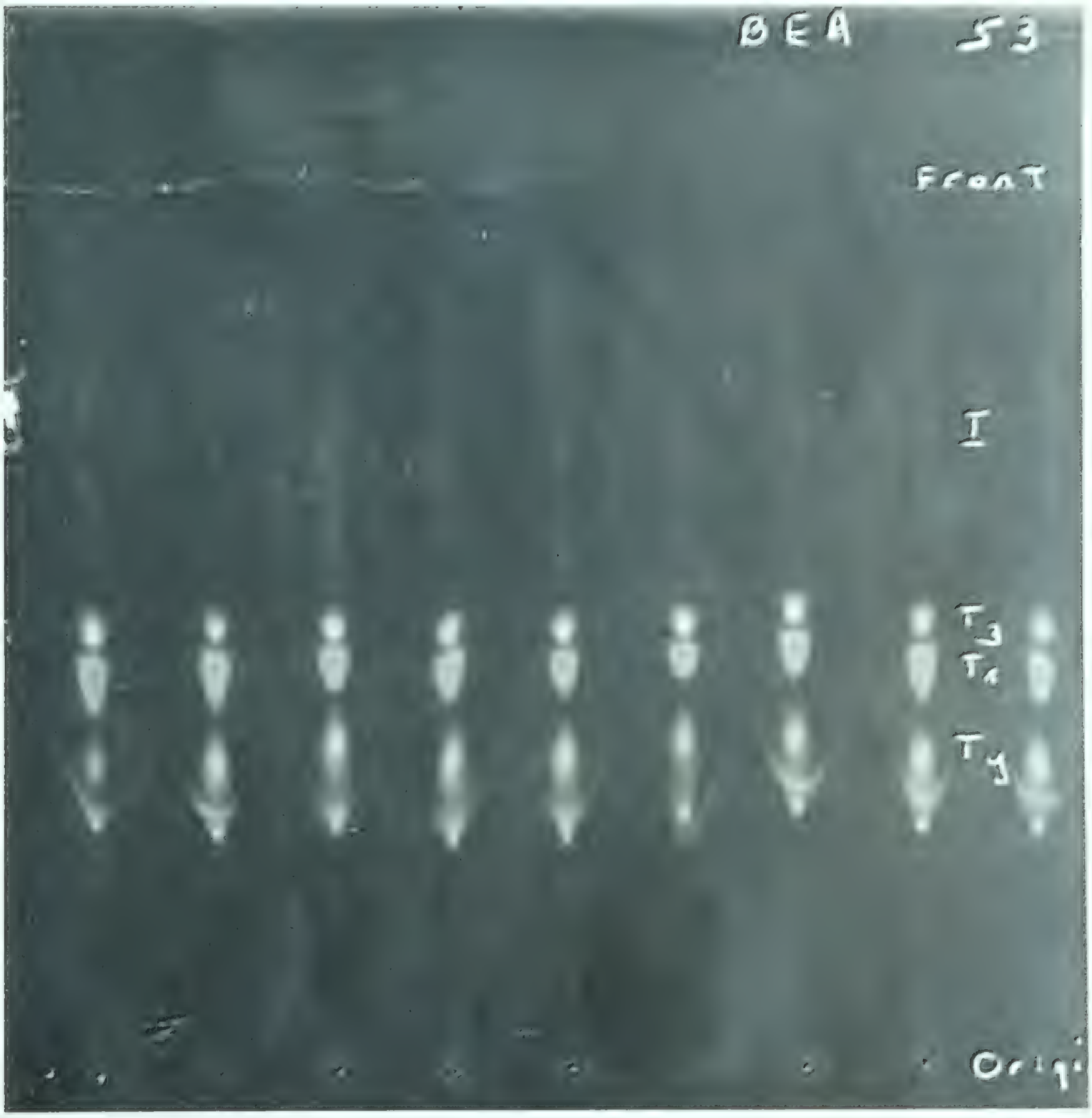
b. Chromatography of untreated plasma

Chromatography of untreated plasma avoids possible artifacts caused by chemical manipulations. Column chromatography, by handling large volumes of plasma, is specially suited for its separation (Robbins et al, 1961). Tong et al (1961) used paper chromatography to separate the iodocompounds of strongly labelled plasma of the cyclostome, *Eptatretus stoutii*.

In this study untreated plasma was separated by thin layer chromatography using silica gel. A relatively large amount of plasma (75-100 μl) could be applied to the silica gel plates and the solvent N butanol:ethanol:6N NH_4OH = 5:2:1 separated the tyrosines, T4, T3 and iodide rapidly and well (Fig. 9). Chavin and Bouwman (1965) used thin layer chromatography with silica gel and 2N butanol:2N NH_4OH:dioxane = 4:5:1 as a solvent to separate the iodocompounds of butanol extracts of goldfish plasma. Hollingsworth et al (1963) used cellulose powder and two satisfactory solvent systems for the separation of iodoamino acid standards.

To insure accurate identification of the circulating iodocompounds in the present study, standards were always chromatogrammed together with plasma samples. It was noticed that radioactive plasma samples were labelling these

Figure 9. Separation of the iodocompounds of plasma on silica gel plate with BEA (butanol:ethanol:6N $NH_4OH = 5:2:1$) as solvent.



standards; in the presence of standards tagged iodoamino acids were present and in the absence of standards the activity was almost entirely limited to the iodide region.

Taurog (1963 c) has shown that air drying of NaI^{131} on filter paper before chromatography results in the appearance of extraneous substances. It was found that NaI^{125} in contact with silica gel behaves in a similar manner (Fig. 1, Fig. 2) and further that exposure to air causes NaI^{125} to label iodoamino acid carriers when in contact with silica gel (Fig. 3, Fig. 4).

KI and the reducing agent MMI (methyl-mercapto-imidazole) eliminate the transformation of NaI^{131} into extraneous substances (Taurog, 1963 c) and the deiodination of labelled iodophenols (Taurog, 1963 a) and thyroid extracts (Taurog, 1963 b). It was found that labelling of iodophenols by NaI^{125} is also stopped by the inclusion of KI or MMI in the sample (Fig. 3, Fig. 4).

Since the circulating iodocompounds are found in very small quantities it is of extreme importance to eliminate labelling of carriers. By including 10^{-3}M MMI in the sample and subjecting the plates to chromatography immediately after sample application, such labelling is absent.

3. Isotopic equilibrium

It was hoped to study the plasma of rainbow trout in a state of isotopic equilibrium. At equilibrium all the iodinated materials in the animal have the same specific activities so that the distribution of radioactivity resembles the stable iodine picture exactly.

Isotopic equilibrium has been achieved in the rat by daily feeding with an I^{131} tagged diet (VanMiddlesworth, 1956), administrating radio iodine daily in the drinking water (Simon and Morel, 1960; Simon, 1963), and by injecting repeatedly with I^{131} (Pitt-Rivers and Rall, 1961). Daily injections of I^{131} were used by Hunn et al (1964) in an attempt to attain isotopic equilibrium in trout.

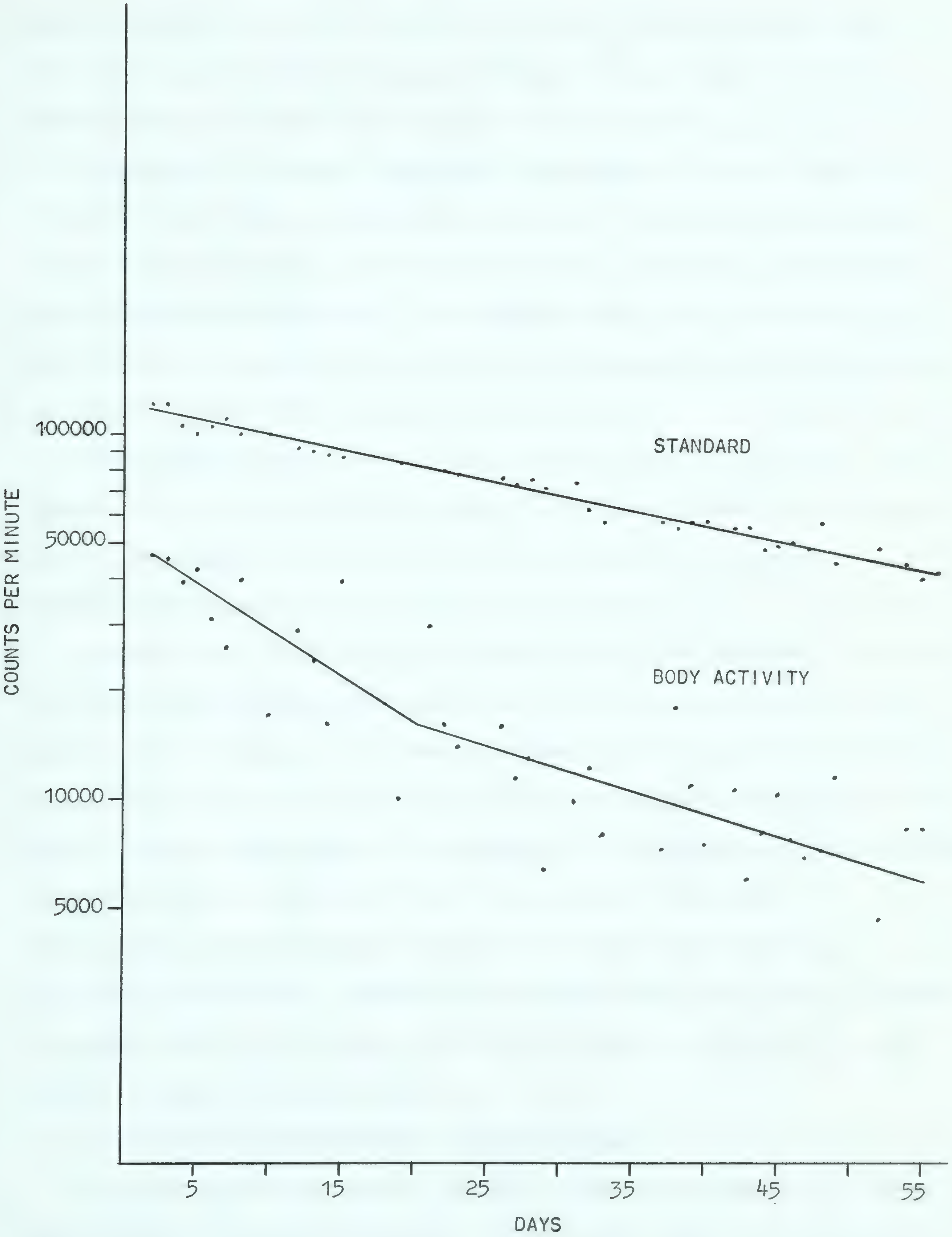
Attempts were first made to achieve isotopic equilibrium in this study by adding I^{125} directly to the aquarium water. It was found that in order to maintain healthy fish the water in the tanks would have to be changed every three or four days. Even then the ammonia level of the water, as determined by reaction with Nessler reagent, rose rapidly in the tanks. Large amounts of I^{125} were necessary to insure plasma samples sufficiently radioactive for analysis. These problems forced the abandonment of this method of administering I^{125} and the alternative method of repeated injections was adopted.

In order to minimize handling and therefore hopefully keep the fish alive for a longer time, injections were spaced three days apart. To check on total body activity, three fish were counted daily together with a standard. A comparison of the loss of activity of the standard and the body activity of the fish of Group A (Fig. 10) shows that the rate of loss of body activity in the latter part of the injection program approximates the loss by physical decay of the standard. This is not true of the early part of the experiment where loss of body activity proceeded at a more rapid rate.

By repeated injections of NaI^{125}, a relatively constant radio iodide supply was made available to the thyroid. With time the hormone produced and released by the gland should contain the same ratio of I^{125} to I^{127} as the iodide entering the thyroid, in other words the specific activities should be the same.

The organic I^{125} level in the blood would be expected to rise as more and more organic I^{125} is released by the thyroid. At first the peripheral utilization of organic I^{125} would be small due to the small proportion of radio organic to stable organic iodine. As the proportion of organic I^{125} increases, its rate of removal from the plasma also increases. Eventually,

Figure 10. Loss of body activity in comparison to physical decay of standard. Experiment A. Body activity = mean of three fish.



100000
50000
10000
5000
COUNTS PER MINUTE
STANDARD
BODY ACTIVITY
5
15
25
35
45
55
DAYS

as the secretion of organic iodine and its exit from the plasma proceed at the same rate, the level of organic I^{125} would remain stable. At this point a state of isotopic equilibrium would be achieved.

Examination of Table 5 and Figure 5 shows that in Group A there is an increase in the organic radio iodine with time. This increase is evident in all three components: the iodotyrosines, T4 and T3. The drop from 50 to 56 days is not significant. The experiment had to be terminated after 56 days and it is impossible to say whether a flattening of the curve was about to begin or whether the rise would have continued.

The hormonal iodine in Group B also shows an increase with time (Table 5). Because of the high iodotyrosine content of the one chromatogram representing day 28, the organic iodine does not show the same gradual increase. No reliable chromatograms were available after 38 days.

McNabb (1963), by stable iodine analysis of one rainbow trout, found the hormonal iodine (2.45 µgm %) to constitute 8.6% of the total iodine (28.36 µgm %). In the present study hormonal iodine rose to a maximum 3.5% and the organic iodine to 4.4% of the total activity. The organic and hormonal iodine did not reach a stable state at the termination of Experiment A and no reliable chromatograms were available for the latter parts of Experiment B. It is likely that a state of isotopic equilibrium had not been reached at the end of 56 days in Experiment A and that the proportion of radio organic to radio inorganic iodine in the plasma would have continued to rise until it represented the stable iodine picture.

4. Circulating iodocompounds in Salmo gairdnerii

T3 was found in the plasma of rainbow trout in approximately the same proportion as T4 (Table 3, Table 4). Leloup identified T3 in the plasma of rainbow trout but found it in smaller quantities than T4 (Leloup and Fontaine,

1960).

In both Groups A and B circulating iodotyrosines were identified and found to contain a maximum of on the average one to one and one-half percent of the total activity. Iodotyrosines are found in the plasma of mammals only in abnormal circumstances. Normally iodotyrosines are deiodinated in the thyroid and not released to the circulation (Pitt-Rivers and Tata, 1959). It is generally assumed that this occurs also in fish but a deiodinase has not been actually demonstrated (Leloup and Fontaine, 1960).

Leloup did not find any iodotyrosines in the plasma of Salmo gairdnerii (Leloup and Fontaine, 1960) but the butanol extraction used preparative to chromatography could possibly explain this. McNabb (1963) concluded from the close agreement of the PBI and BEI determinations of the plasma of one rainbow trout, that very small amounts of iodotyrosines are found in the plasma in comparison to the hormonal iodine. More trout PBI and BEI values should be compared to determine whether this trout was typical. It is possible that iodotyrosines may not be represented in the PBI value due to lack of binding by plasma proteins or a type of binding similar to that of iodide in salmonids, which is broken by trichloracetic acid precipitation.

The absolute quantities of iodocompounds found in the plasma at a given time give no information as to the rate of secretion of the compounds into the blood or their peripheral utilization. Approximately equal amounts of T4 and T3 were found in the plasma of rainbow trout. It is unlikely that these substances are secreted into and disappear from the plasma at equal rates. T4 is bound more strongly to plasma proteins than is T3 (Leloup and Fontaine, 1960) and would therefore be expected to penetrate the cells more slowly than T3. If the disappearance rate of T3 is more rapid than that of T4 and the absolute quantity of the two compounds in the plasma at a given

time is equal, it follows that more T3 than T4 is secreted by the thyroid gland.

One quarter to one third of the organic iodine in rainbow trout was found to be iodotyrosine. Again, nothing can be concluded concerning the secretion rate, peripheral degradation or excretion of the iodotyrosines. The iodotyrosines may be secreted in large amounts and may undergo rapid degradation or excretion or the opposite may be the case.

B. THYROID IODOCOMPOUNDS

All vertebrate thyroids concentrate iodide and produce MIT, DIT and T4 (Gorbman, 1955). Iodide is oxidized to iodine and incorporated into tyrosine molecules, probably bound in thyroglobulin moecules, to form MIT and DIT. Coupling of two DIT molecules yields T4 and one DIT and MIT molecule yield T3 (Pitt-Rivers and Tata, 1959; Leloup and Fontaine, 1960).

MIT, DIT, T4, T3 and iodide have been identified by chromatography in the thyroids of several species of teleosts (Berg et al, 1959). In this study these compounds were also found but a large proportion of the activity of the thyroid hydrolyzates could not be identified (Fig. 6, Table 6). The unknown separated into two distinct peaks after chromatography in BEA and was spread over a large area after phenol chromatography (Fig. 6). It is thus probable that more than one compound was involved in the unknown.

Increased digestion with pancreatin did not reduce the proportion of the unknown material (Table 6). One of the unknown peaks remained at the origin after BEA chromatography but the unknown migrated a considerable distance from the origin after phenol chromatography. It is thus unlikely that the unknown was unhydrolyzed thyroglobulin.

Unknown compounds have been identified in the thyroids of several species of fish, but usually in small quantities (Berg et al, 1959). Tong et al (1961)

analyzed thyroids of the hagfish, *Eptatretus stoutii*, under conditions similar to those employed in this study, (extraction with 0.9% NaCl and digestion with pancreatin). 18-37% of the activity was found at the origin after chromatography. This unknown material was not reduced by further digestion with pancreatin nor by digestion with papain. Hydrolysis with 8% $Ba(OH)_2$ released inorganic radio iodine. The work on the hagfish and the present study of rainbow trout differ from other studies in not employing butanol extraction procedures. Perhaps the unknown material is insoluble in butanol or is changed in some way by butanol extraction.

By repeated injections, a relatively constant supply of radio iodide was made available to the thyroid. The radio iodide was incorporated into MIT, DIT, T4 and T3 and the distribution of activity among these compounds did not change much with time. It is thus likely that isotopic equilibrium had been achieved in the thyroid gland. Figure 7 would then represent the distribution of the stable as well as the radioactive iodocompounds in the thyroid gland.

Iodide is apparently quickly incorporated into the thyroglobulin molecules in the thyroid. The release of iodothyronines into the circulation seems to be rather slow however as evidenced by the length of time necessary to attain isotopic equilibrium in the plasma.

C. TISSUE PENETRATION OF IODIDE AND IODIDE BINDING

Eales (1963 b) states that, since as far as inorganic iodine is concerned a state of equilibrium between the plasma and the cells is quickly established, a change in the T/P ratio would imply a change between the blood and tissue of an organic radio iodine fraction. After an injection of I^{131} the T/P ratios increased in steelhead trout corresponding to the appearance of hormonal iodine in the blood and its assumed availability to

the tissues. The increase was greatest in the metabolically active tissues brain, gut, liver and kidney (Eales, 1963 b).

The T/P values of Group A remain quite stable. In Group B however the T/P of liver, kidney, skin and, to a smaller extent, gut rise gradually (Fig. 8). It is unlikely that an increase in the hormonal iodine is the explanation for the rising ratios observed in Experiment B. The greatest rise in the T/P values occurred between days 10 and 19 and the circulating hormonal iodine remained quite constant at this time (Table 5). Moreover, the rise in plasma hormonal iodine evident in Experiment A is not reflected in any marked change in T/P values.

The low penetration of iodine into the tissues demonstrated in this study contrasts markedly to the rapid and complete penetration of iodine shown by Hickman (1959) in marine starry flounder. Leloup and Fontaine (1960) have shown that in the salmonids iodide is largely bound to plasma proteins. This binding is evidenced in the low penetration of iodide into the red blood cells of these species. Red blood cells of Salmo gairdnerii suspended in saline had a T/P ratio of 0.65. The ratio decreased as plasma was substituted for the saline and when plasma alone was present the T/P ratio was 0.23. Binding of iodide to plasma was further indicated when equilibrium dialysis showed that a drop of I^{131} was concentrated to a far greater extent in the plasma of rainbow trout than in saline. Electrophoresis of plasma to which I^{131} had been added showed two peaks of radioactivity, one in the free iodide zone and one in the albumin zone, again indicating bound iodide in the rainbow trout (Leloup and Fontaine, 1960). This iodide binding likely prevents iodide from penetrating into the cells of rainbow trout to any great extent.

The significantly higher T/P ratios of Group B (Fig. 10) point to a greater penetration of radio iodine into the tissues of this group. In

Group B a large dose of stable iodine was injected with the radio iodine. Perhaps the iodide binding sites in the plasma were saturated to a greater extent in this case. More iodine would then be free and available for entry into the cells and higher T/P values would result. The fluctuations in T/P values in Experiment B may be the result of differences in free iodine availability in the plasma.

The saturation of binding sites by stable iodide in iodine-rich situations could also explain the increased excretion of radio iodine found by Leloup and Fontaine (1960) in rainbow trout in iodine-enriched water. These workers found that Salmo gairdnerii in iodine-enriched water excreted 68.4% of the injected I^{131} dose in 24 hours while trout in untreated water excreted 33.5% of the dose.

In the absence of any binding of iodide it would be expected that the addition of stable iodine to the environment or to the circulation would slow the removal rate of radio iodine. In the presence of large quantities of stable iodine, a smaller proportion of the iodine atoms will be tagged and the proportional removal of radio/stable iodine will decrease even though the absolute quantity of iodine excreted may be greatly increased. This is supported by a study of radio iodine excretion rates of starry flounder in natural and iodine-enriched fresh water (Hickman, 1959). Contrary to this expectation, Berg and Gorbman (1953) found that radio iodine was excreted more rapidly by platyfish in iodine-rich water than by fish in normal water. It is not known whether iodide binding occurs in this species.

It was found that in Experiment B of the present study, where a relatively high dose of NaI^{127} was injected with the NaI^{125}, higher levels of organic iodine appeared in the plasma in the early stages of the experiment than in Experiment A (Table 5). McNabb (1963) found that the hormonal secretion rate of rainbow trout in iodine-enriched water was only slightly higher than the

rate in control fish. Different rates of excretion of radio iodine in Groups A and B offer a more likely explanation of the higher percentages of hormonal iodine in Group B. In the fish of Group B a larger percentage of the injected radio iodine would have been excreted at the time of sampling than in Group A. The organic iodine secreted by the thyroid, in being more firmly bound to plasma proteins, would not be excreted at the same rapid rate. If roughly the same amounts of hormone are secreted by the thyroids of Groups A and B, the proportion of hormone in the plasma of the fish of Group B would then be higher due to the greater excretion of iodide in this group.

V. SUMMARY

1. NaI^{125} in contact with silica gel and exposed to air undergoes transformation reactions that result in the appearance of extraneous compounds.

2. NaI^{125} in contact with iodoamino acid standards on silica gel and exposed to air transfers part of its radioactivity to these standards. Inclusion of 10^{-3}M KI or 10^{-3}M MMI (methyl-mercapto-imidazole) in the sample effectively stabilizes the NaI^{125}.

3. Iodotyrosines, T4, T3 and iodide are found in the plasma of *Salmo gairdnerii*. T4 and T3 are found in approximately equal amounts and in this study account for a maximum 3.5% of the activity while the iodotyrosines share a maximum 1-1.5% of the activity. A state of isotopic equilibrium was probably not reached in the plasma after 56 days of injection with NaI^{125}.

4. Thyroid hydrolyzates of *Salmo gairdnerii* contain MIT, DIT, T4, T3, iodide and a large proportion of unknown material. Prolonged digestion of thyroid extracts does not decrease the proportion of unknown material. Isotopic equilibrium is reached more quickly in the thyroid gland than in the plasma.

5. Penetration of radio iodine into the tissues is greater when the stable iodine content of the plasma is increased. It is proposed that a greater saturation of the iodide binding sites (Leloup and Fontaine, 1960) allows more free iodide to cross the cell boundaries.

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